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Communication

P450BM3-Catalyzed Oxidations Employing Dual Functional Small Molecules

Sébastien J.-P. Willot¹, Florian Tieves¹, Marco Girhard² , Vlada B. Urlacher²,
Frank Hollmann^{1,*}  and Gonzalo de Gonzalo^{3,*} ¹ Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629HZ Delft, The Netherlands² Institute of Biochemistry, Heinrich-Heine University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany³ Departamento de Química Orgánica, Universidad de Sevilla, c/ Profesor García González 1, 41012 Sevilla, Spain* Correspondence: F.Hollmann@tudelft.nl (F.H.); gdegonzalo@us.es (G.d.G.);
Tel.: +31-15-2781957 (F.H.); +34-954-559997 (G.d.G.)

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Abstract: A set of dual functional small molecules (DFSMs) containing different amino acids has been synthesized and employed together with three different variants of the cytochrome P450 monooxygenase P450BM3 from *Bacillus megaterium* in H₂O₂-dependent oxidation reactions. These DFSMs enhance P450BM3 activity with hydrogen peroxide as an oxidant, converting these enzymes into formal peroxygenases. This system has been employed for the catalytic epoxidation of styrene and in the sulfoxidation of thioanisole. Various P450BM3 variants have been evaluated in terms of activity and selectivity of the peroxygenase reactions.

Keywords: biocatalysis; cytochrome P450; dual functional small molecules; epoxidations; sulfoxidation

1. Introduction

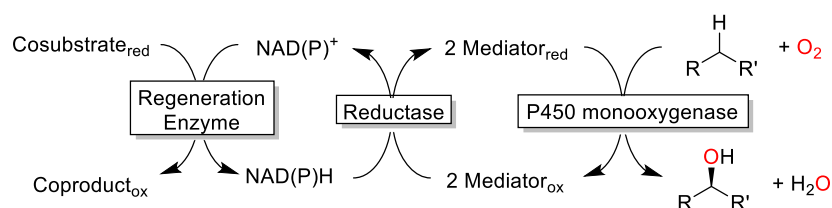
Cytochrome P450 monooxygenases (P450 or CYP) catalyze a broad range of oxyfunctionalization reactions of non-activated C–H- and C=C-bonds [1–6]. Especially, the frequently observed regio- and enantioselectivity of this transformation makes P450s potentially very useful tools in preparative biocatalysis [7].

The catalytic cycle of P450 monooxygenases comprises the reductive activation of molecular oxygen to form the catalytically active oxyferryl species (i.e., a highly oxidized iron-oxo-complex). The reducing equivalents needed for this reaction are generally derived from reduced nicotinamide cofactors via more or less complex electron transport chains [8], adding complexity to the reaction schemes [9].

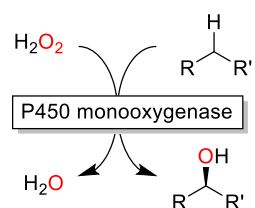
In 1999, Arnold and coworkers proposed to preparatively exploit the well-known hydrogen peroxide shunt pathway [10]. Here, the catalytically active compound is formed directly from H₂O₂ thereby drastically simplifying the regeneration scheme of P450 monooxygenases (Scheme 1).

Unfortunately, the majority of the known P450s are rapidly inactivated by H₂O₂ making the H₂O₂ shunt pathway practically irrelevant. Some exceptions are known, in which P450s can efficiently use H₂O₂ through a substrate-assisted reaction mechanism for the hydroxylation or decarboxylation of fatty acids [11–15].

A) P450 monooxygenase-catalysed oxyfunctionalisation following the classical regeneration scheme:

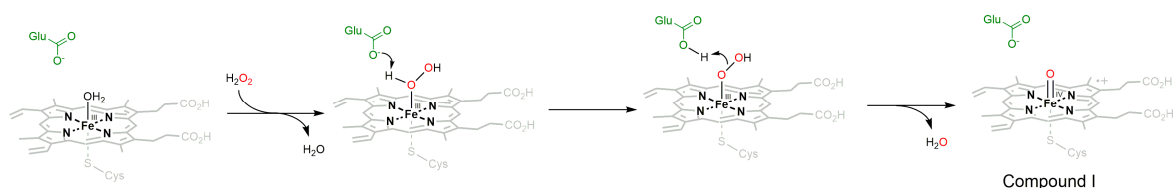


B) P450 monooxygenase-catalysed oxyfunctionalisation using the H_2O_2 -shunt pathway:



Scheme 1. Comparison of the classical regeneration and the H_2O_2 -shunt pathway to drive P450 monooxygenase-catalyzed oxyfunctionalization reactions.

Recently, Cong and coworkers reported an elegant possible solution to the H_2O_2 -related inactivation of P450 monooxygenases [16,17]. By comparing the catalytic mechanism and active sites of P450 monooxygenases with those of (H_2O_2 -dependent) peroxygenases, these authors reasoned that a base (Glutamate) present in peroxygenases but missing in the active site of P450 monooxygenases may account for the poor activity of P450 monooxygenases with H_2O_2 (Scheme 2).



Scheme 2. Formation of Compound I from H_2O_2 in peroxygenases. The active-site base glutamate (Glu, green) facilitates the reaction by first deprotonating the primary H_2O_2 -adduct and by reprotonation of the peroxo-intermediate.

To alleviate this shortcoming, a range of base-modified decoy molecules was suggested. In essence, these dual functional small molecules (DFSMs) comprise an imidazole-base coupled via a linker moiety to an amino acid anchoring part in order to position the base within the P450 monooxygenases' active sites, thereby enabling peroxygenase-like reactions [18,19]. In the current study, we set out to validate and broaden this very interesting concept.

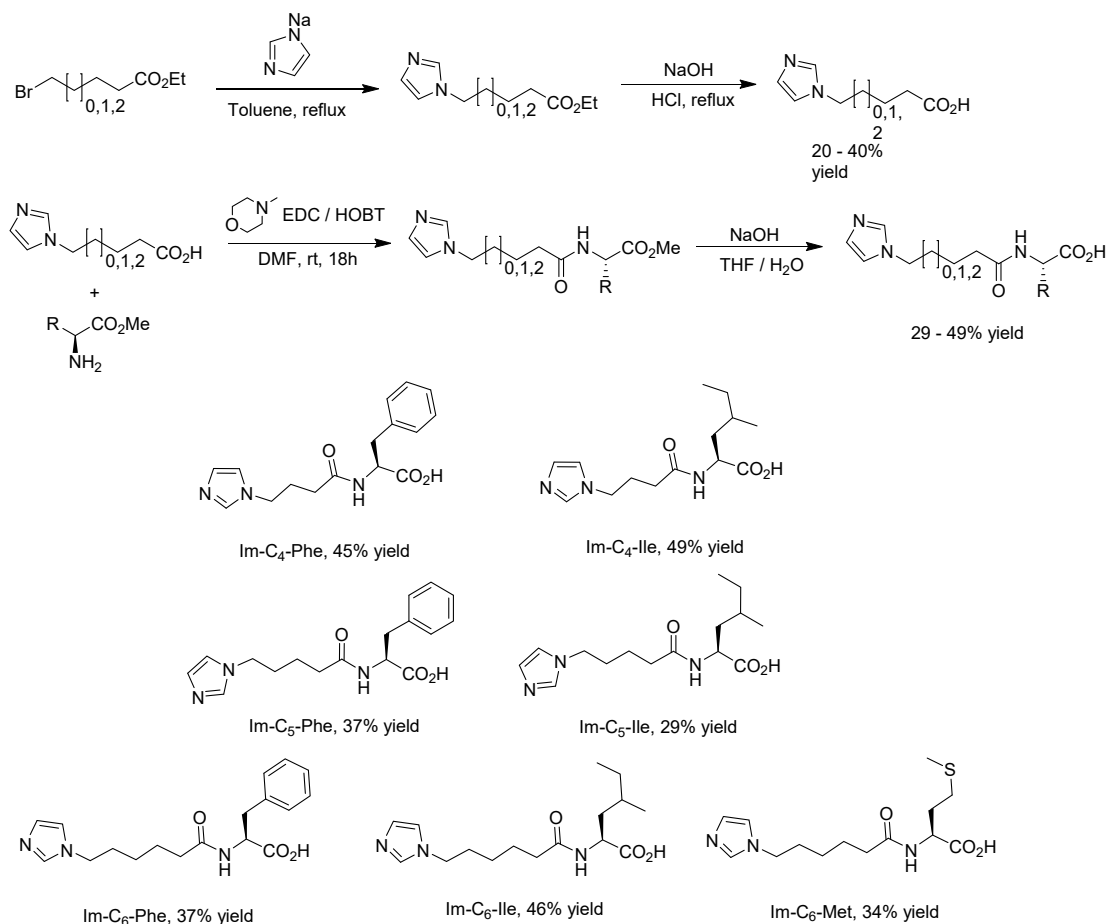
2. Results

2.1. Preparation of the Dual Functional Small Molecules (DFSMs)

Imidazole-based dual DFSMs were synthesized following a literature-known four-step procedure [17,20]. Overall, seven DFSMs comprising different amino acids and different spacer lengths were synthesized (Scheme 3). It should be mentioned here that amongst the seven DFSMs synthesized only 3 (Im-C5-Ile, Im-C6-Phe and Im-C6-Ile) showed significant activity with the enzyme tested.

For the P450 monooxygenase we chose the well-known CYP102A1 (P450BM3) from *Bacillus megaterium*. More specifically, three variants P450BM3 F87A, P450BM3 V78A/F87A and P450BM3 A74E/F87V/P386S were recombinantly expressed in *Escherichia coli* and purified following literature methods [21,22]. All variants contained a mutation at position 87, which had previously been reported to broaden the substrate scope of P450BM3 [23]. The side-chain of phenylalanine 87 extends into

the lumen of the substrate access channel close to the heme iron and thus residues with less bulky side-chains, such as mutations to alanine or valine, widen the access channel by creating incremental space in the vicinity of the heme iron [23]. The mutation V78A has a similar effect, making the hydrophobic pocket that encloses the heme iron more capacious than in the wild type [23]. The variant P450BM3 A74E/F87V/P386S has previously been shown to possess 2 or 2.5 fold increased catalytic activity for the oxidation of β -ionone compared to the F87A or F87V single variants, respectively, and was therefore also included here [21].



Scheme 3. Dual Functional Small Molecules (DFSMs) synthesized for the P450BM3-catalyzed oxidations.

2.2. Biocatalytic Transformations Using the DFSMs/P450BM3 System

Having all catalytic components at hand, we first investigated the influence of the DFSMs on the P450BM3-catalyzed and H_2O_2 -driven epoxidation of styrene (**1**) to obtain optically active styrene oxide (**2**). As shown in Table 1, only three of the seven DFSMs enabled H_2O_2 -driven reactions with P50BM3.

Pleasingly, we found that the presence of DFSMs significantly improved the catalytic performance of all P450BM3 variants. In case of the F87A variant for example, Im-C6-Phe increased the product formation almost 20 fold. Other combinations gave similar improvements. However, at present time we are unable to rationalize the improvements in light of DFSM binding to the enzyme active site and/or positioning of the substrates. Further studies will be necessary to obtain a quantitative structure–activity relationship. In line with the pH optimum of P450BM3 [24], the highest turnover numbers were observed at slightly alkaline pH values (Table 1, entries 1 vs. 5 and 6; entries 9 vs. 12). Decreasing the H_2O_2 concentration appeared to have a positive effect on the product formation (Table 1, entries 1 vs. 7), which we attribute to a lower inactivation rate at lower peroxide concentrations.

Interestingly, the DFSMs also influenced the enantioselectivity of the epoxidation reaction, which is in line with the original report by Cong and coworkers [17]. Possibly, this is due to a more

stringent positioning of the starting material in the enzyme active site. However, again, no obvious structure–activity relationship was observed.

Table 1. Epoxidation of styrene (1) catalyzed by DFSM/P450BM3 using H₂O₂ as the oxidant.^a

C=Cc1ccccc1 (1) + H₂O₂ $\xrightarrow{\text{P450BM3 / DFSM}}$ C1OC1Cc2ccccc2 (R)-2 + H₂O

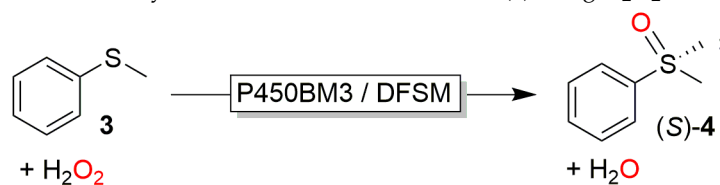
Entry	DFSM	pH	c(2) (μM)	ee (%) ^b
F87A variant				
1	Im-C6-Phe	8	117	72
2	Im-C6-Ile	8	94	59
3	Im-C5-Ile	8	58	54
4	None ^c	8	6	10
5	Im-C6-Phe	7	68	65
6	Im-C6-Phe	6	≤2	–
7	Im-C6-Phe ^d	8	133	77
V78A/F87A variant				
8	Im-C6-Phe	8	54	6
9	Im-C6-Ile	8	97	22
10	Im-C5-Ile	8	80	19
11	None	8	11	n.d.
12	Im-C6-Ile	6	≤2	n.d.
A74E/F87V/P386S variant				
13	Im-C6-Phe	8	≤2	n.d.
14	Im-C6-Ile	8	17	4 (S)
15	Im-C5-Ile	8	17	6 (S)
16	None	8	≤2	n.d.

^a Reaction conditions: P450BM3 (0.5 μM), styrene (4 mM), H₂O₂ (20 mM; except entry 7), DFSM (0.5 mM), in phosphate buffer. Reaction time: 30 min; ^b Determined by Gas Chromatography; ^c No reaction was observed in absence of DFSM or biocatalyst; ^d c(H₂O₂) = 5 mM; n.d. not determined.

Similarly, P450BM3-catalyzed sulfoxidation of thioanisole (3) was positively influenced by DFSMs (Table 2). Compared to the epoxidation reaction, rate accelerations were somewhat lower; the enantioselectivity of the sulfoxidation reaction, however, was significantly improved by the DFSMs. Both observations can be rationalized by the spontaneous (non-enantioselective) oxidation of thioanisole by H₂O₂ [25]. Quite interestingly, the P450BM3 A74E/F87V/P386S variant, which in the epoxidation reaction gave rather poor results compared to the other two variants, excelled in the sulfoxidation reaction.

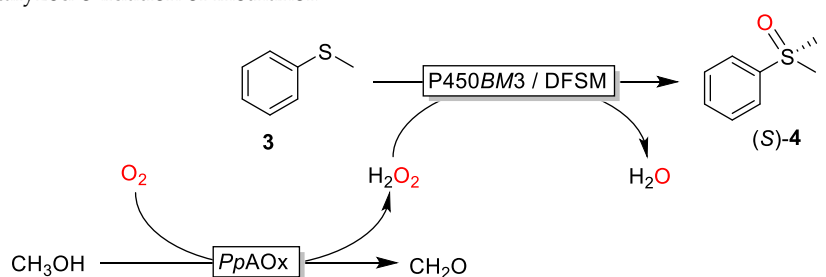
As mentioned above, H₂O₂-related inactivation of the heme enzyme appeared to be a major limitation of the proposed H₂O₂-shunt pathway reaction of P450BM3. We therefore also investigated the effect of controlled in situ H₂O₂ generation via reductive activation of O₂ using an oxidase [26]. Thus, employing the commercially available alcohol oxidase from *Pichia pastoris* (PpAOx), H₂O₂ was generated in situ from O₂ at the expense of methanol (which was oxidized to formaldehyde).

When this system was applied (Table 3), reaction rates were significantly decreased (reaction times 18 h), while at the same time the turnover numbers of the biocatalyst were improved, compared to the use of H₂O₂ they were five times greater. The low concentration of H₂O₂ available slowed down both the reaction rate and the oxidative inactivation. We expect that further optimized reaction schemes may provide optimal H₂O₂ generation rates, ensuring maximized enzymatic sulfoxidation while minimizing the H₂O₂-related inactivation of the heme enzyme. Again, in the absence of any DFSM, near racemic product was observed, indicating predominant spontaneous sulfoxidation.

Table 2. P450BM3-catalyzed sulfoxidation of thioanisole (**3**) using H₂O₂ as the oxidant.^a

Entry	DFSM	c(4) (μM)	ee (%) ^b
F87A variant			
1	Im-C6-Phe	232	40
2	Im-C6-Ile	178	38
3	Im-C5-Ile	151	35
4	None	78	6
V78A/F87A variant			
5	Im-C6-Phe	103	41
6	Im-C6-Ile	75	40
7	Im-C5-Ile	77	44
8	None	53	4
A74E/F87V/P386S variant			
9	Im-C6-Phe	129	47
10	Im-C6-Ile	98	51
11	Im-C5-Ile	108	53
12	None	65	8

^a Reaction conditions: P450BM3 (0.5 μM), thioanisole (4 mM), H₂O₂ (20 mM), DFSM (0.5 mM), in phosphate buffer. Reaction time: 30 min; ^b Determined by Gas Chromatography.

Table 3. P450BM3 F87A-catalyzed sulfoxidation of **3** using in situ generation of H₂O₂ by the PpAOx-catalyzed oxidation of methanol.^a

Entry	DFSM	c(4) (μM)	ee (%) ^b
1	Im-C6-Phe	381	31
2	Im-C6-Ile	359	33
3	Im-C5-Ile	320	30
4	None	248	≤3

^a Reactions were stopped after 18 h at room temperature. For further reaction details, see Materials and Methods section; ^b Determined by Gas Chromatography.

One major drawback of classic P450 monooxygenation reactions is that, due to the exclusive water solubility of the nicotinamide cofactors, they have to be performed in aqueous reaction media. As the majority of the reagents of interest for P450 monooxygenase-catalyzed oxyfunctionalizations are rather hydrophobic, reagent concentrations tend to be in the lower millimolar range, reducing the preparative attractiveness of these reactions from an economic and environmental point-of-view [27]. In this respect, the proposed peroxide-driven reaction offers some interesting possibilities for non-aqueous reactions using P450 monooxygenases.

To test this hypothesis, we evaluated the epoxidation of styrene using precipitated P450BM3 F87A suspended in neat styrene as the reaction medium; the stoichiometric oxidant was *t*BuOOH (Scheme 4).



Scheme 4. P450BM3-catalyzed epoxidation in neat styrene using *t*BuOOH as stoichiometric oxidant.

Very pleasingly, under these conditions, epoxidation of styrene was observed in the presence of Im-C5-Ile (TON_{P450BM3} = 178, TON = [Styrene oxide]/[P450BM3]), while in the absence of Im-C5-Ile, 100 turnovers were still observed for the biocatalyst. (*R*)-2 was obtained with optical purities around 15% for both reactions. In the absence of the P450BM3 F87A variant, no product formation was observed, even upon prolonged reaction times. To the best of our knowledge, this is the first example of a P450 monooxygenase reaction under neat conditions.

3. Materials and Methods

Unless otherwise noted, analytical grade solvents and commercially available reagents were used without further purification.

Dual functional small molecules (DFSMs) were synthesized according to the methodology described in the literature [17]. Compounds Im-C5-Ile, Im-C5-Phe, Im-C6-Ile, Im-C6-Phe and Im-C6-Met exhibited physical and spectral properties in accordance with those reported [17].

GC (Gas Chromatography) analyses were performed on a Shimadzu GC-2010 Plus (Shimadzu, Kyoto, Japan). For the oxidation of styrene (**1**) to styrene oxide (**2**), a ChirasilDex CB (Agilent, Santa Clara, CA, USA, 25 m × 0.32 mm × 0.25 μm) column was employed: Carrier gas He, 100 °C hold 12.50 min, 20 °C min⁻¹ to 225 °C, hold 1 min. Retention times: **1**: 3.0 min; (*R*)-**2**: 7.5 min; (*S*)-**2**: 7.9 min and dodecane (internal standard): 9.8 min. For the oxidation of thioanisole (**3**) to methyl phenyl sulfoxide (**4**) a Lipodex E (Agilent, 50 m × 0.25 mm × 0.25 μm) column was used: Carrier gas He, 130 °C hold 6.0 min, 20 °C min⁻¹ to 200 °C, hold 5.0 min, 25 °C min⁻¹ to 220 °C hold 1.0 min. Retention times: **3**: 4.2 min; dodecane (internal standard): 4.9 min; (*S*)-**4**: 11.8 min, and (*R*)-**4**: 12.4 min.

NMR Spectra were recorded (¹H NMR 300 MHz; ¹³C NMR 75 MHz) with the solvent peak used as the internal reference (7.26 and 77.0 ppm for ¹H and ¹³C, respectively) using an Agilent 400 (400 MHz, Santa Clara, CA, USA)

P450BM3 F87A and P450BM3 V78A/F87A were produced according to a previously reported protocol [22].

3.1. Preparation of P450BM3 A74E/F87V/P386S

pET28a P450BM3 A74E F87V P386S was expressed in *E. coli* BL21 (DE3). First, 5 mL Lysogeny Broth (LB) medium supplemented with 30 μg mL⁻¹ kanamycin was inoculated with single colonies and grown overnight at 37 °C, 180 rpm for preculture. Then 1 L medium (900 mL Terrific Broth (TB) + 100 mL phosphate buffer pH 7.5) supplemented with kanamycin (30 μg mL⁻¹) was inoculated with the 5 mL preculture and incubated at 37 °C, 180 rpm. When OD₆₀₀ reached 0.8 (OD₆₀₀: Absorbance at 600 nm), induction was obtained by adding isopropyl β-D-1-thiogalactopyranoside (100 μmol L⁻¹); at this timepoint 5-aminolevulinic acid (500 μmol L⁻¹) FeSO₄ (100 μmol L⁻¹) were also added. Cultures were then stirred at 30 °C, 180 rpm overnight. Cells were harvested by centrifugation (10,000 g, 30 min, 4 °C) and resuspended in 50 mM phosphate buffer pH 7.5 supplemented with RNase and DNase. After 30 min on ice, cells were disrupted with a French press. The cells debris was removed by centrifugation (14,000 rpm, 30 min, 4 °C). Purification was performed by nickel affinity chromatography using a 60 mL His-Trap FF crude column (GE Healthcare, Chicago, IL, USA) applying a gradient of

imidazole. The enzyme was then desalted with a PD-10 column and concentrated with an Amicon filter with a cut-off of 30 kDa.

3.2. General Procedure for the Preparation of Im-C4-Phe and Im-C4-Ile

Im-C4-Phe and Im-C4-Ile were prepared starting from 4-(1H-imidazol-1-yl)butanoic acid: A DMF (Dimethylformamide) solution (10 mL) containing HOBt (150 mg, 1.1 mmol), EDC (170 mg, 1.1 mmol), and 4-(1H-imidazol-1-yl)butanoic acid (154 mg, 1.0 mmol) was stirred at room temperature for 1 h. A solution of L-phenylalanine methyl ester or L-isoleucine methyl ester (1.1 mmol) and 4-methylmorpholine (202 mg, 2.0 mmol) dissolved in 10 mL of DMF was then added to the reaction mixture. After 18 h, the reaction mixture was partitioned between dichloromethane (50 mL) and H₂O (50 mL). The organic layer was washed with H₂O (3 × 50 mL) and dried over MgSO₄. The solution was concentrated under reduced pressure. The crude product was then dissolved in 2 mL NaOH aqueous solution (1.0 M) and 1 mL THF and stirred overnight. The THF was removed under reduced pressure and the solution was acidified to pH 2.0 with HCl (1.0 M). Water was then removed under reduced pressure and the residue was dissolved in ethanol. NaCl was separated by filtration and ethanol was evaporated to give the final products.

(S)-2-(4-(1H-imidazol-1-yl)butanamido)-3-phenylpropanoic acid (Im-C4-Phe): Colorless oil (135.4 mg, 45% yield). ¹H-NMR (300 MHz, *d*₆-DMSO): δ (ppm) 8.97 (s, 1H), 8.13 (d, 1H, *J* = 8.0 Hz), 7.91 (s, 1H), 7.62 (s, 1H), 7.31–7.14 (m, 5H), 4.37–4.29 (m, 1H), 4.13 (t, 2 H, *J* = 7.5 Hz), 3.07–3.01 (m, 1H), 2.82–2.74 (m, 1H), 2.07–1.96 (m, 4H). ¹³C-NMR (75.4 MHz, *d*₆-DMSO): δ (ppm) 174.2, 172.6, 138.3, 135.6, 129.2 (2C), 128.7, 126.6 (2C), 124.8, 121.2, 54.1, 48.9, 37.1, 35.2, 25.3. HRMS: *m/z* calculated for C₁₆H₁₉N₃O₃ (M⁺): 302.1499; found: 302.1498.

(S)-2-(4-(1H-imidazol-1-yl)butanamido)-3-methylpentanoic acid (Im-C4-Ile): Colorless oil (137.7 mg, 49% yield). ¹H-NMR (300 MHz, *d*₆-DMSO): δ (ppm) 9.12 (s, 1H), 8.19 (s, 1H), 7.95 (s, 1H), 7.67 (s, 1H), 4.16–4.11 (m, 1H), 3.92 (t, 2H, *J* = 7.9 Hz), 2.14–2.07 (m, 4H), 1.93–1.87 (m, 2H), 1.21–1.13 (m, 3H), 0.89–0.80 (m, 6H). ¹³C-NMR (75.4 MHz, *d*₆-DMSO): δ (ppm) 174.1, 171.3, 137.6, 128.6, 119.8, 57.2, 49.0, 36.2, 34.4, 32.1, 31.9, 25.2, 16.1, 11.8. HRMS: *m/z* calculated for C₁₄H₂₃N₃O₃ (M⁺): 281.1739; found: 281.1734.

3.3. General Procedure for the Biocatalyzed Oxidation of Styrene and Thioanisole Employing the P450BM3/DFSM System

Unless otherwise stated, the corresponding variant of P450BM3 (0.5 μM) was transferred to a glass sample bottle containing 0.1 M, pH 8.0 phosphate buffer (0.36 mL), styrene (**1**) or thioanisole (**3**) (4 mM in methanol) and the DFSM (0.5 mM, dissolved in pH 8.0 phosphate buffer). H₂O₂ (20 mM, dissolved in pH 8.0 phosphate buffer) was added and the reaction was shaken at room temperature and 300 rpm for 30 min. The reaction was then extracted using ethyl acetate containing 5.0 mM of dodecane as the external standard (0.4 mL) and dried over anhydrous sodium sulfate. The conversion and the optical purity of styrene oxide (**2**) or methyl phenyl sulfoxide (**4**) was analyzed by gas chromatography.

3.4. General Method for the Biocatalyzed Oxidations Employing the H₂O₂ In Situ Generation System

P450BM3 F87A (0.5 μM) was transferred to a glass sample bottle containing 0.1 M, pH 8.0 phosphate buffer (0.36 mL), methanol (100 mM), thioanisole (**3**) (4 mM in methanol) and the DFSM (0.5 mM, dissolved in pH 8.0 phosphate buffer). A solution of the alcohol oxidase from *Pichia pastoris* (5 nM, dissolved in pH 8.0 phosphate buffer) was added and the reaction was shaken at room temperature and 300 rpm for 18 h. The reaction was then extracted using ethyl acetate containing 5 mM of dodecane as the external standard (0.4 mL) and dried over anhydrous sodium sulfate. The conversion and the optical purity of methyl phenyl sulfoxide (**4**) was analyzed by gas chromatography.

3.5. General Method for the Epoxidation of Styrene Using Precipitated P450BM3 F87A with DFSMs

P450BM3 F87A was precipitated with acetone and dried (30 mg) and transferred to a glass sample bottle containing styrene (**1**) (200 μ L) and the DFSM (0.5 mM, dissolved in pH 8.0 phosphate buffer). t BuOOH (20 mM, 70% in H₂O) was added and the reaction was shaken at room temperature and 300 rpm for 30 min. The reaction was then extracted using ethyl acetate containing 5.0 mM of dodecane as the external standard (0.4 mL) and dried over anhydrous sodium sulfate. The conversion and the optical purity of styrene oxide (**2**) was analyzed by gas chromatography.

4. Conclusions

Overall, we have confirmed Cong's approach, turning P450 monooxygenases into peroxygenases by using DFSMs. The results shown in this study suggest specific interactions of the DFSMs with the enzymes (here P450BM3) influencing their performance as co-catalysts. Further studies with a broader set of DFSMs will be necessary to establish quantitative structure–activity relationships and further optimize the reaction system. It will also be interesting to investigate possible match/mismatch combinations of the (chiral) amino acid anchoring groups.

One exciting possibility of DFSMs arises from the fact that P450 monooxygenase catalysis becomes independent from (exclusively water soluble) nicotinamide cofactors and thereby enables the use of P450 monooxygenases under neat reaction conditions.

Author Contributions: G.d.G. and F.H. devised the experiments, S.J.-P.W. and F.T. performed the experiments and analyzed the experimental data, M.G. and V.B.U. provided resources and contributed to the experimental design. All authors contributed to the data interpretation and composition of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available.



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